11-1-2015

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Cassandra DeMarshall

Min Han

Eric Nagele

Abhirup Sarkar

Nimish Acharya

See next page for additional authors

Immunology Letters 168 (2015) 80–88

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

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Cassandra A. DeMarshall[a,b,c], Min Han[a,b,c], Eric P. Nagele[a,e], Abhirup Sarkar[a,b,c], Nimish K. Acharya[a,c], George Godsey[b,c], Eric L. Goldwaser[a,b,c], Mary Kosciuk[a,c], Umashanger Thayasivam[d], Benjamin Belinka[e], Robert G. Nagele[a,c,e,*], On behalf of: The Parkinson's Study Group Investigators

[a] Biomarker Discovery Center, New Jersey Institute for Successful Aging, Rowan University School of Osteopathic Medicine, Stratford, NJ, USA
[b] Graduate School of Biomedical Sciences, Rowan University, Stratford, NJ, USA
[c] Department of Geriatrics and Gerontology, Rowan University School of Osteopathic Medicine, Stratford, NJ, USA
[d] Department of Mathematics, Rowan University, Glassboro, NJ, USA
[e] Durin Technologies, Inc., New Brunswick, NJ, USA

ARTICLE INFO

Article history:
Received 11 June 2015
Received in revised form
14 September 2015
Accepted 14 September 2015
Available online 16 September 2015

Keywords:
Parkinson's disease
Autoantibodies
Diagnosis
Protein microarrays
Biomarkers

ABSTRACT

Introduction: There is a great need to identify readily accessible, blood-based biomarkers for Parkinson's disease (PD) that are useful for accurate early detection and diagnosis. This advancement would allow early patient treatment and enrollment into clinical trials, both of which would greatly facilitate the development of new therapies for PD.

Methods: Sera from a total of 398 subjects, including 103 early-stage PD subjects derived from the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) study, were screened with human protein microarrays containing 9,486 potential antigen targets to identify autoantibodies potentially useful as biomarkers for PD. A panel of selected autoantibodies with a higher prevalence in early-stage PD was identified and tested using *Random Forest* for its ability to distinguish early-stage PD subjects from controls and from individuals with other neurodegenerative and non-neurodegenerative diseases.

Results: Results demonstrate that a panel of selected, blood-borne autoantibody biomarkers can distinguish early-stage PD subjects (90% confidence in diagnosis) from age- and sex-matched controls with an overall accuracy of 87.9%, a sensitivity of 94.1% and specificity of 85.5%. These biomarkers were also capable of differentiating patients with early-stage PD from those with more advanced (mild-moderate) PD with an overall accuracy of 97.5%, and could distinguish subjects with early-stage PD from those with other neurological (e.g., Alzheimer's disease and multiple sclerosis) and non-neurological (e.g., breast cancer) diseases.

Conclusion: These results demonstrate, for the first time, that a panel of selected autoantibodies may prove to be useful as effective blood-based biomarkers for the diagnosis of early-stage PD.

1. Introduction

Currently, there are no simple and reliable diagnostic tests for Parkinson's disease (PD). It remains essentially a clinical diagnosis, subject to variations in patient presentation and physician awareness. Even in subjects with an apparently positive response to dopaminergic medication, a clinical diagnosis of PD can have relatively poor accuracy [1]. Results are worse for early-stage PD subjects [2]. Neuroimaging approaches such as dopamine transporter (DaT) scanning have some utility, but are expensive, invasive, and lack specificity. Thus, there remains a great need for an accurate, inexpensive, and noninvasive test that can detect early-stage PD.

Recently, many laboratories have been investigating new diagnostic strategies that are focusing on protein, lipid and microRNA biomarkers that are detectable in the blood in response to the

* Corresponding author at: Department of Geriatrics and Gerontology, New Jersey Institute for Successful Aging, Rowan University School of Osteopathic Medicine, 2 Medical Center Drive, Stratford, NJ 08084, USA.
E-mail address: nagelero@rowan.edu (R.G. Nagele).

http://dx.doi.org/10.1016/j.imlet.2015.09.010

presence of disease [3–5]. For example, in the field of Alzheimer's disease, a number of studies have identified panels of these blood-based biomarkers that may be useful for detection of this disease which are currently under development [3,6,7]. Comparable developments for biomarkers of PD have lagged behind. Detection of biomarkers in the cerebrospinal fluid (CSF) or blood presumably associated with PD pathogenesis, such as alpha-synuclein or DJ-1, have thus far failed to yield consistent results [8–12]. Our previous studies have shown that autoantibodies should be added to the list of blood proteins that have potential as useful biomarkers of disease [13,14]. These autoantibodies are abundant and ubiquitous in the blood, and their levels are influenced by a variety of factors including age, gender, and the presence of disease [15–17]. In previous studies, we have identified autoantibodies in human sera that can serve as biomarkers to diagnose mild-moderate stages of PD with an overall accuracy of 97.1%, with comparable results also obtained for mild-moderate Alzheimer's disease (AD) [13,14].

Our earlier success at identifying specific autoantibody biomarkers capable of detecting mild-moderate PD prompted the present study, the goal of which was to determine if a separate panel of autoantibodies can also be used for accurate detection and diagnosis of early-stage PD. To test this possibility, sera from a total of 398 subjects, including 103 early-stage PD subjects from the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) study with 90% confidence in diagnosis, were analyzed with human protein microarrays to identify useful autoantibody biomarkers for early-stage PD. These biomarkers were evaluated for their ability to distinguish early-stage PD subjects from controls, from individuals with mild-moderate PD and from individuals with other neurodegenerative and non-neurodegenerative diseases.

2. Methods

2.1. Ethics statement

Approval for use of serum samples in this study was obtained from the Rowan-Stratford Institutional Review Board.

2.2. Participants

103 early-stage PD samples from subjects participating in the DATATOP study were obtained in coordination with the Michael J. Fox Foundation and Parkinson Study Group. These came from subjects participating in the DATATOP study, which was a clinical trial investigating the potential beneficial effects of two anti-oxidative therapies, deprenyl and tocopherol, with the goal of delaying the time at which patients progress to disability requiring levodopa treatment [18]. Diagnosis of PD was made with follow-up at 90% confidence by DATATOP clinical investigators, with each patient determined to have a Hoehn and Yahr scale score ranging from 1 to no greater than 2 [18]. Twenty-nine mild-moderate PD and 50 mild-moderate Alzheimer's disease serum samples were obtained from Analytical Biological Systems, Inc. (Wilmington, DE). Thirty stages 0-2 breast cancer (BC) serum samples were obtained from Asterand, Inc. (Detroit, MI), and 30 multiple sclerosis (MS) patient serum samples were obtained from BioServe Biotechnologies Ltd. (Beltsville, MD). Healthy age- and sex-matched control sera were obtained from several sources: 40 from Analytical Biological Systems, Inc., 65 from BioServe Biotechnologies Ltd., 28 from Asterand Inc., and 23 from The New Jersey Institute for Successful Aging at Rowan University (Stratford, NJ). All samples were handled using standard procedures and stored at −80 °C until use. Demographic characteristics of the study population are displayed in Table 1.

2.3. Human protein microarrays

To identify autoantibodies in human sera, we used *Invitrogen's* ProtoArray v5.0Human Protein Microarrays (Cat. *No.* PAH0525020, *Invitrogen*, Carlsbad, CA, USA), each containing 9,486 unique human protein antigens (www.invitrogen.com/protoarray). Arrays were probed with serum diluted 1:500 and scanned to detect the AlexaFluor 647 reporter (*Cat.No. A-21445, Invitrogen*) according to the manufacturer's instructions using a GenePix 4000B Fluorescence Scanner (*Molecular Devices*, Sunnyvale, CA, USA).

2.4. Microarray data analysis

Fluorescence data was acquired by aligning the *Genepix Array List* (GAL) onto the microarray using *Genepix Pro* analysis software. The resulting *Genepix Results* (GPR) files were imported into Invitrogen's *Prospector 5.2* for analysis and biomarker selection. The "group characterization" and "two – group comparison" features in the *IRBP Toolbox* within *Prospector* then enabled M-statistical analysis of differential autoantibody expression between the two subjects groups. Positive hits were determined by a Z-Factor greater than 0.4, and a minimum signal intensity of 1500 RFU, which allow for stringent biomarker selection and minimizes the amount of false positives. Autoantibodies were sorted into descending order by difference of prevalence between early-stage PD and control groups, and the top 50 most differentially expressed autoantibodies were chosen as our selected panel of diagnostic biomarkers. All raw data has been deposited for public access in a MIAME compliant database (GEO) under accession number GSE62283.

The predictive classification accuracy of the selected biomarkers in the Training Set, Testing Set, and both sets combined was tested with *R's Random Forest* (*RF*) (v 4.6-10), using default settings [19–21]. Selected biomarkers were tested with the *RF* model algorithm, and classification accuracy is reported in a confusion matrix. Receiver operating characteristic curves (ROCs), widely used to evaluate the utility of a diagnostic test, were generated using R(3.02) packages ROCR(v 1.0-5) and pROC(v 1.7.3) [6,22].

3. Results

3.1. Selection of a panel of autoantibody biomarkers for early-stage PD diagnosis

Previously published data from our laboratory has highlighted the potential utility of autoantibodies as blood-based biomarkers for diagnosing mild-moderate PD [13]. Here, we used the same strategy to search for biomarkers useful for early-stage PD detection (Fig. 1). Early-stage PD serum samples ($n = 103$) from patients with a clinical diagnosis of PD at 90% confidence and 111 age- and sex- matched control samples (total $n = 214$) (Table 1) were randomly separated into Training and Testing Sets. The Training Set samples were used only for biomarker selection and contained 52 early-stage PD samples and 56 controls. The Testing Set samples, which were used to evaluate the performance of the selected biomarkers, were not involved in the biomarker selection process and included 51 early-stage PD and 55 control samples. Human protein microarrays containing 9486 antigens were probed with Training or Testing Set sera. Using *Prospector* analysis software, 2470 autoantibodies with a significantly ($p < .05$) higher prevalence in the early-stage PD group compared to controls were identified in the Training Set as potential diagnostic biomarkers. From this list, the top 50 most differentially expressed autoantibodies in the early-stage PD subject group were chosen as a working diagnostic panel of biomarkers (Supplementary Table 1).

Table 1
Subject demographics. For each disease group the number of individuals (*n*), age, range of age, gender, and ethnicity are listed. For the early-stage PD subjects, the Unified Parkinson's Disease Rating Scale (UPDRS) and Hoehn and Yahr scores are included as indices of PD severity.

Group	*n*	Age		Sex	Ethnicity	UPDRS	Hoehn & Yahr
		(Years)	(Range)	(% Male)	(% Caucasian)		
Parkinson's disease	132	65.1 ± 10.3	37–88	57	89	–	–
-Early-stage	103	62.7 ± 9.3	37–79	58	98	38.1 ± 16.8	2.1 ± 0.6
-Mild-moderate	29	74.3 ± 9.0	53–88	55	55	–	–
Controls	156	55.0 ± 15.6	19–87	56	76	–	–
-Age-matched	111	63.1 ± 8.4	51–87	56	78	–	–
-Non-age-matched	45	34.9 ± 10.2	19–50	49	71		
Alzheimer's disease	50	78.5 ± 8.8	61–97	42	88	–	–
Multiple sclerosis	30	51.0 ± 9.2	36–67	33	97	–	–
Breast cancer	30	46.9 ± 5.8	32–54	0	97	–	–

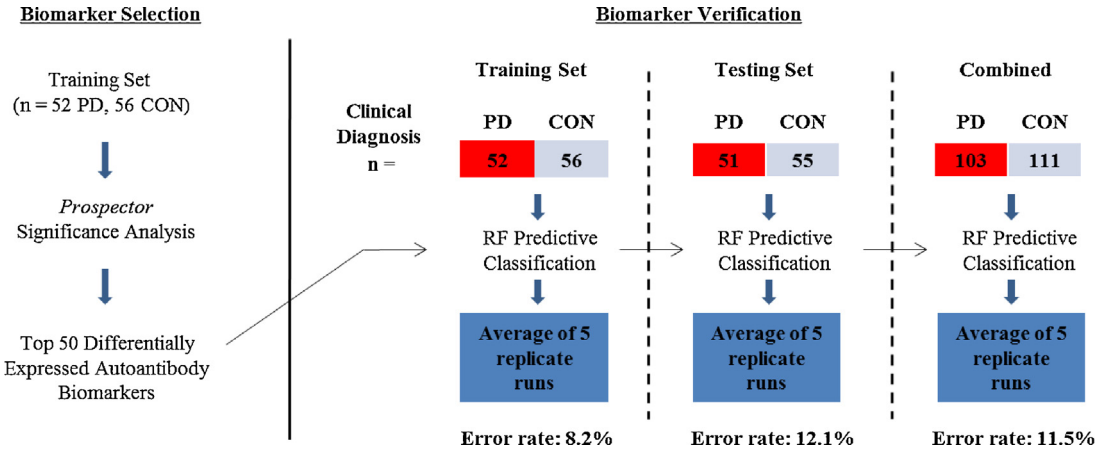

Fig. 1. Biomarker selection and Training/Testing Set analysis strategy. The total sample pool (*n* = 214) was randomly split into two groups: a Training Set and Testing Set. *Prospector* statistical analysis was performed on the Training Set to identify the top 50 most differentially expressed autoantibody classifiers in early-stage PD samples compared to controls. The diagnostic accuracy of these selected biomarkers was tested by using *Random Forest* to predict sample classification in the Training Set. This logic was then applied to the analysis of the Testing Set, and then both sets combined. The values represent an average of five runs for each of the three steps of the biomarker discovery and validation process.

Table 2
a and b. Diagnostic results using panels of 50 and four early-stage PD biomarkers, respectively. The performance of the top 50 and top four early-stage PD autoantibody biomarkers was assessed using *RF*. Using the original logic, *RF* successfully distinguished the early-stage PD samples of the Testing Set (*n* = 51) from age- and sex-matched controls, age-matched plus younger controls, mild-moderate PD, mild-moderate AD, multiple sclerosis and breast cancer with high overall accuracies. PPV, positive predictive value; NPV, negative predictive value.

(a)

Early-stage PD (*n* = 51) vs.

	Age-matched controls	Mild-moderate PD	Mild-moderate AD	Multiple sclerosis	Breast cancer
n	55	29	50	30	30
Sensitivity%	94.1	96.1	94.1	94.1	96.1
Specificity%	85.5	100.0	100.0	100.0	100.0
PPV%	85.7	100.0	100.0	100.0	100.0
NPV%	94.0	93.6	94.3	90.9	93.8
Overall accuracy%	87.9	97.5	97.0	96.3	97.5
Overall error%	12.1	2.5	3.0	3.7	2.5

(b)

Early-stage PD (*n* = 51) vs.

	Age-matched controls	Mild-moderate PD	Mild-moderate AD	Multiple sclerosis	Breast cancer
n	55	29	50	30	30
Sensitivity%	84.3	90.2	92.2	84.3	90.2
Specificity%	83.6	100.0	100.0	100.0	96.7
PPV%	82.7	100.0	100.0	100.0	97.9
NPV%	85.2	85.3	92.6	79.0	85.3
Overall accuracy%	84.0	93.7	96.0	90.1	92.6
Overall error%	16.0	6.3	4.0	9.9	7.4

3.2. Verification of biomarkers via training and testing set analyses

The top 50 autoantibody biomarkers chosen from the Training Set serum samples as the early-stage PD diagnostic panel were then re-verified as significant predictors by *Random Forest (RF)*. Upon evaluation of Training Set samples (*n* = 108; 52 early-stage PD, 56 controls) utilizing these 50 biomarkers, early-stage PD subjects were distinguished from age- and sex-matched controls with

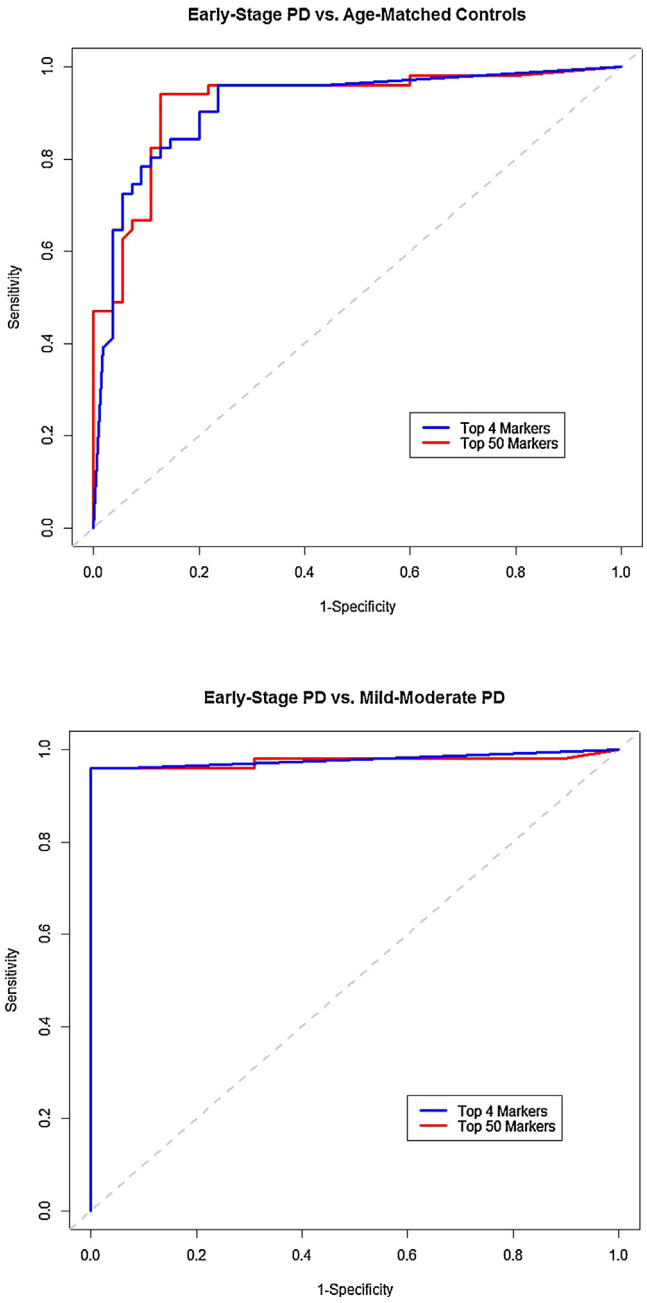

Fig. 2. (a and b) ROC assessment of autoantibody biomarkers for detection of early-stage PD in Testing Set subjects and PD progression. a. Comparison of early-stage PD ($n = 51$) vs. age-matched controls ($n = 55$) using a panel of 50 (red line) or 4 (blue line) PD biomarkers shows that these biomarker panels can be used to detect early-stage PD with relatively high overall accuracy. The dashed line represents the line of no discrimination. b. Comparison of early-stage Testing Set PD subjects ($n = 51$) vs. mild-moderate PD subjects ($n = 29$) using a panel of 50 (red line) or 4 (blue line) early-stage PD biomarkers shows that these biomarkers can be used to accurately distinguish these two different stages of PD progression. The ROC AUC, sensitivity, and specificity values for the 50 and 4 biomarkers used for this purpose are shown in Table 3.

Table 3
ROC curve analysis of diagnostic results using the early-stage PD Testing Set. ROC curve analysis was used to assess the diagnostic utility of the panels of 50 and four selected biomarkers for distinguishing early-stage PD subjects from age-matched controls from the subject groups listed. Areas under the curve (AUC) at 95% confidence are listed along with values for sensitivity and specificity derived from the ROC curve output data.

	50 Markers			4 Markers		
Early-stage PD ($n = 51$) vs.	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Age-matched controls $n = 55$)	0.92(0.87, 0.98)	0.94(0.88, 1.0)	0.87(0.78, 0.94)	0.91(0.86, 0.96)	0.84(0.74, 0.94)	0.87(0.76, 0.90)
Mild-moderate PD ($n = 29$)	0.97(0.94, 1.0)	0.96(0.90, 1.0)	1.0(1.0, 1.0)	0.98(0.95, 1.0)	0.96(0.90, 1.0)	1.0(1.0, 1.0)
Mild-moderate AD ($n = 50$)	0.99(0.97, 1.0)	0.93(0.89, 0.98)	0.96(0.90, 1.0)	0.98(0.94, 1.0)	0.96(0.9, 1.0)	0.98(0.94, 1.0)
Multiple sclerosis ($n = 30$)	0.97(0.94, 1.0)	0.96(0.90, 1.0)	1.0(1.0, 1.0)	0.97(0.94, 1.0)	0.96(0.90, 1.0)	1.0(1.0, 1.0)
Breast cancer ($n = 30$)	0.98(0.95, 1.0)	0.96(0.90, 1.0)	0.97(0.9, 1.0)	0.98(0.95, 1.0)	0.96(0.97, 1.0)	0.97(0.90, 1.0)

an average of 91.8% prediction accuracy. We then used the same 50 biomarkers and Training Set logic for unbiased classification of early-stage PD in Testing Set samples which were not involved in biomarker selection. *RF* correctly classified early-stage PD in Testing Set subjects (n =n106; 51 early-stage PD, 55 controls) with an average accuracy of 87.9% (Table 2a). Diagnostic sensitivity, specificity, and positive and negative predictive values for the panel of 50 early-stage PD biomarkers using only Testing Set subjects are shown in Table 2a. The diagnostic utility of this biomarker panel for distinguishing early-stage PD Testing Set subjects from age-matched controls was also evaluated using Receiver Operating Characteristic (ROC) curve analysis [23] (Fig. 2a, red line). The ROC area under the curve (AUC) for this comparison was 0.92, indicating excellent classification accuracy (Table 3). Finally, combining Training and Testing Set samples (total n = 214), *RF* successfully distinguished early-stage PD from controls with an average overall accuracy of 88.5%.

3.2. Minimum number of autoantibodies required for accurate diagnosis of early-stage PD

To determine the minimum number of autoantibody biomarkers required to achieve the best diagnostic accuracy, the 50 selected early-stage PD biomarkers were first sorted according to decreasing relative importance, and then successively removed from the bottom of the list until the overall diagnostic accuracy began to decline significantly. Using this approach, we determined that a panel of four biomarkers (the top four biomarkers listed in Supplementary Table 1) was the minimum number required to maintain an effective diagnostic accuracy (ROC AUC = 0.91; sensitivity = 0.84; specificity = 0.87) for distinguishing early-stage PD subjects from age-matched controls (Fig. 2a, blue line; Table 3).

3.3. Specificity of the selected biomarker panels for early-stage PD

The specificity of the selected autoantibody biomarker panels for early-stage PD was tested to determine if they could distinguish early-stage PD from other neurological and non-neurological diseases. To eliminate the possibility that the selected PD biomarker panels were simply detecting non-specific CNS degeneration, the same 51 early-stage PD sera from Testing Set subjects were compared to sera from 50 patients with Alzheimer's disease (AD) and 30 sera from patients with multiple sclerosis (MS). Using the panel of 50 biomarkers, early-stage PD sera were readily distinguished from AD sera with an overall accuracy of 97.0% (sensitivity = 94.1%; specificity = 100.0%) (Table 2a). ROC curve analysis yielded an AUC of 0.99 and a sensitivity and specificity of 0.93 and 0.96, respectively (Table 3). Using the panel of four biomarkers yielded an overall accuracy of 96.0% (ROC AUC = 0.98; sensitivity = 0.96; specificity = 0.98) (Tables 2 b and 3). Both biomarker panels were capable of readily distinguishing early-stage PD from MS subjects with comparable overall accuracy (Tables 2 a,b and 3).

We then sought to determine the specificity of the early-stage PD diagnostic biomarkers in the context of non-neurological disease, in this case breast cancer. Results showed that 30 breast cancer (BC) serum samples were successfully differentiated from the 51 early-stage, Testing Set PD samples with an overall accuracy of 97.5% (sensitivity = 96.1%; specificity = 100.0%; ROC AUC = 0.98) for the panel of 50 biomarkers (Tables 2 a and 3). ROC analysis using the panel of four biomarkers yielded an overall accuracy of 92.6%, an AUC of 0.98, a sensitivity of 0.96, and specificity of 0.97 (Tables 2 b and 3). These results demonstrate that the panels of 50 and four PD autoantibody biomarkers were comparably accurate in distinguishing early-stage PD subjects from patients with other neurological and non-neurological diseases.

3.4. Use of PD biomarkers to distinguish early- from later-stages of PD progression

We next asked whether the panels of 50 and four early-stage PD biomarkers could distinguish early-stage PD from later stages with more advanced pathology. To address this, the 51 early-stage, Testing Set PD serum samples were compared to 29 mild/moderate-stage PD samples using *RF*. Early-stage PD samples were correctly classified with an overall accuracy of 97.5% (sensitivity = 96.1%; specificity = 100.0%) (Table 2a) and ROC AUC of 0.97 (Fig. 2b, red line; Table 3) using the panel of 50 biomarkers. Comparable results were obtained with the panel of four early-stage PD biomarkers (Fig. 2b, blue line; Table 3). These findings emphasize the high level of specificity of these biomarker panels for the diagnosis of early-stage PD, and also suggest the potential utility of this approach for identifying discrete stages of PD disease progression as illustrated in Fig. 3. Lastly, comparison of the top 50 biomarkers for early-stage and mild-moderate PD revealed an overlap of 21 biomarkers (Supplementary Table 2), confirming the expected presence of common biomarkers between these disease stages.

3.5. Effects of addition of younger controls on biomarker selection and diagnostic accuracy

There is a growing realization that most CNS diseases are preceded by long prodromal phases of ongoing, gradually escalating pathology for many years prior to the emergence of detectable symptoms [24–27]. This reality makes it very difficult for samples derived from our aging population to be both age-matched and unequivocally pathology-free. To investigate the impact of this potential limitation, we tested the effects of including younger, non-age-matched controls on the initial selection of early-stage PD autoantibody biomarkers. To accomplish this, 52 early-stage PD samples were compared to a control group composed of 56 age-matched and 45 additional younger, non-age-matched controls. As described above, the top 50 early-stage PD autoantibody biomarkers were selected in *Prospector* on the basis of prevalence difference between the two groups. The utility of these 50 new biomarkers for distinguishing early-stage PD (n = 103) subjects from age-matched controls (n = 111) was then verified using *RF,* yielding an overall diagnostic accuracy of 88.8%, a sensitivity of 93.2% and specificity of 84.7%. When all early-stage PD samples (n = 103) were compared to all age-matched and non-age-matched controls (n = 156; 111 age-matched, 45 non-age-matched) these biomarkers yielded an overall diagnostic accuracy of 90.4%, a sensitivity of 94.2%, and a specificity of 87.8%. These results were comparable to those obtained using the original 50 biomarkers described above. This is not surprising considering that, among the 50 new early-PD biomarkers, 32 were found to overlap with the previous set of 50 biomarkers that were derived from inclusion of only age- and sex-matched controls, and the identities of the four top biomarkers remained unchanged (Fig. 4).

4. Discussion

Our previous studies have shown that autoantibodies are abundant and ubiquitous in human sera, numbering in the many thousands, and that individual autoantibody profiles are strongly influenced by age, gender and the presence of disease [13,14,16]. Further, differential autoantibody expression profiles can be used to detect and diagnose mild-moderate PD and AD using a small volume of serum or plasma and human protein microarrays as a diagnostic platform [13,14]. In the present study, we sought to extend the capacity and potential clinical utility of this approach by attempting to identify autoantibody biomarkers that can diagnose

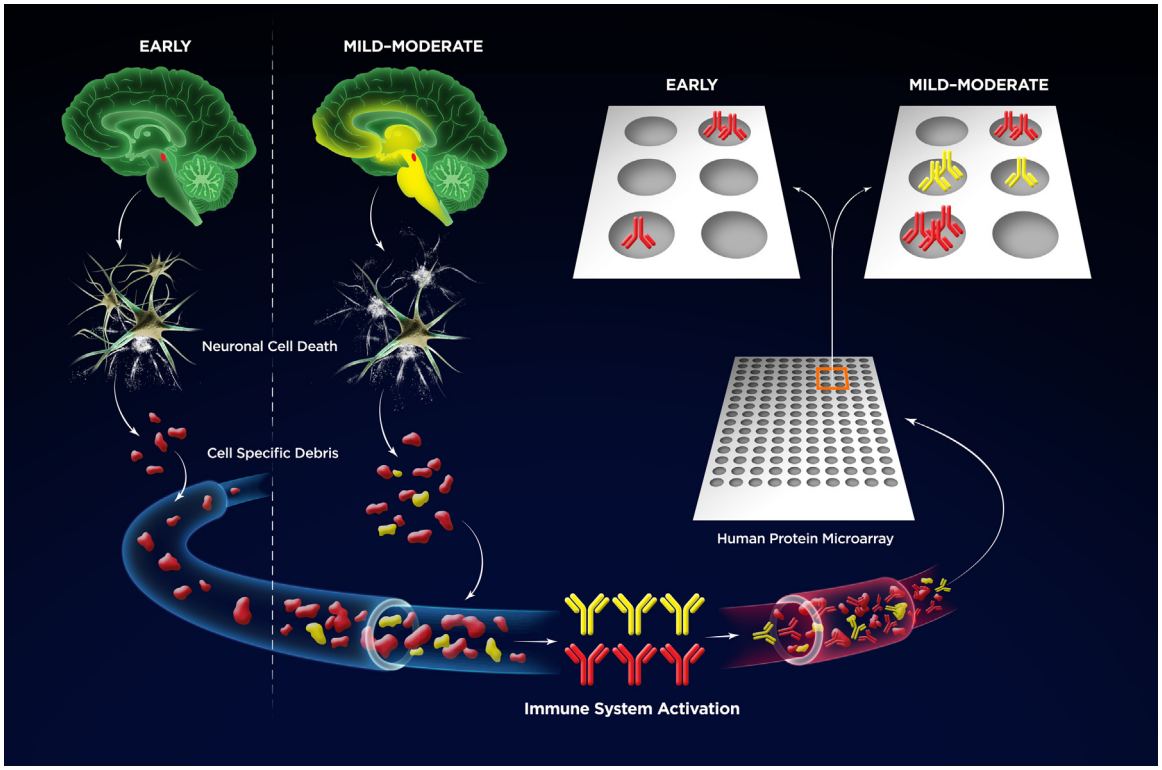

Fig. 3. Utility and hypothetical origin of autoantibodies useful for PD diagnosis and staging. It is well-known that by the time symptoms emerge at early-stage PD, the majority of neurons in the substantia nigra (red dot in brain) have already died, and it is assumed that their debris (red particles) is liberated into the surrounding brain tissue. Some of this debris makes its way into the blood, activates the immune system, and elicits the production of corresponding autoantibodies. In early-stage PD, the site of pathology and debris production is more focal, and the spectrum of disease-associated autoantibodies is likewise limited (red autoantibodies). It is reasonable to expect that escalation and spreading of PD pathology during later disease stages (e.g., mild-moderate PD) leads to more abundant and complex (red and yellow) debris and disease-associated (red and yellow) autoantibody profiles. For each disease stage, autoantibodies exhibiting the most dramatic and consistent changes are selected as the useful biomarkers.

early-stage PD as well as distinguish early- from mild-moderate-stage PD.

To accomplish this, we obtained sera from subjects enrolled in the DATATOP study through the Michael J. Fox Foundation and Parkinson's Study Group. These subjects were diagnosed initially and later confirmed at subsequent patient visits with early-stage PD with at least 90% confidence based on functional, motor, cognitive, and psychiatric assessments [18]. The DATATOP clinical trial was aimed at testing the potential beneficial effects of two antioxidative therapies, deprenyl and tocopherol, on the progression of PD. These agents were found to have no discernable beneficial effects on PD progression [18]. Autoantibody biomarker profiles for DATATOP subjects with early-stage PD were obtained and compared with age- and sex-matched controls as well as with sera from PD patients with more advanced (mild-moderate) disease. We initially identified a panel containing the top 50 PD autoantibody biomarkers that was able to distinguish early-stage PD subjects from age- and sex-matched controls. Concerning the top 50 chosen biomarkers, it is important to point out that the majority are expressed in both early-stage PD and controls; however, those in early-stage PD subjects demonstrate significant fold increases in expression compared to corresponding controls. We then verified their significance and predictive value using an independent Testing Set containing subjects that were not involved in the biomarker discovery process. Results showed an accuracy for early-stage PD detection of 87.9%, a sensitivity of 94.1% and a specificity of 85.5%. ROC curve assessment of the utility of the diagnostic yielded an AUC of 0.92 with 50 biomarkers and 0.91 with four biomarkers. Since it is generally considered desirable for a diagnostic test to have a sensitivity and specificity greater than 85%, the two biomarker panels for early-stage PD detection described here exceed these criteria for the specific population studied [28]. Among the 50 PD autoantibody biomarkers identified, the top four represent the minimum number required for accurate detection and diagnosis of early-stage PD. Moreover, both panels of biomarkers are specific in differentiating early-stage PD from other neurological and non-neurological diseases, such as AD, MS, and breast cancer.

The panels of 50 and four autoantibody biomarkers described here have also allowed us to distinguish early-stage PD from mild-moderate PD with overall accuracies of 97.5% (sensitivity = 96.1%, specificity = 100.0%) for the 50 biomarkers and 93.7% (sensitivity = 90.2%, specificity = 100.0%) for the four biomarkers. ROC curve analysis yielded an AUC of 0.97 for the 50 biomarkers and 0.98 for the four biomarkers for this application. A diagnostic test capable of distinguishing different stages of PD severity may make it possible to follow a patient's disease course, rate of progression, and response to therapies. This would be useful for physicians and their patients as well as for enabling early enrollment of PD subjects into clinical trials and monitoring of therapeutic efficacy through a patient's response to new treatments. Of course, for the latter, a positive patient response would be a delay of disease progression to the next stage or an improvement from the current disease state as evidenced by curtailed symptoms. Any slowing or stopping of disease progression resulting from diminished pathology would be expected to be accompanied by a corresponding reduction in levels of disease-associated autoantibodies (Fig. 3).

It is widely recognized that the pathogenesis of a number of neurodegenerative diseases is initiated many years prior to the emergence of clinically useful symptoms. For effective and accurate identification of biomarkers directly linked to pathology, the selection of truly pathology-free controls is just as important as the selection of subjects with confirmed pathology. However,

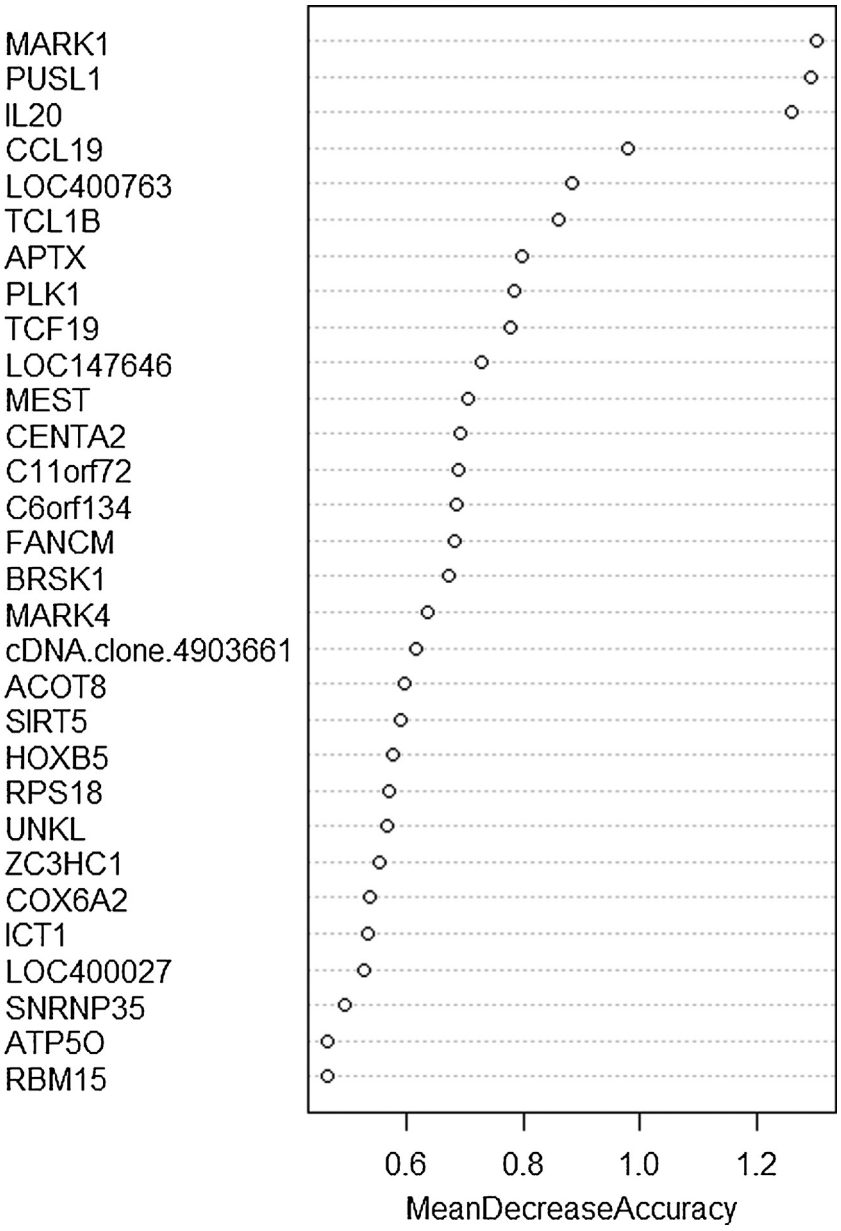

Fig. 4. Effect of younger controls on biomarker selection and diagnostic importance. Gini plot demonstrating the relative importance of 30 of the top 50 biomarkers to the *RF* classification decision when comparing early-stage PD to age-matched controls plus younger healthy controls. Biomarkers are sorted according to decreasing relative importance from top to bottom, with the relative impact of each biomarker to the *RF* classification decision indicated by the extent of deflection of the indicator point to the right side of the plot. Note that the identities of the top 4 biomarkers remain unchanged.

without the aid of telltale symptoms during prodromal phases of disease, it is difficult to ensure that age-matched controls being used for biomarker discovery are truly pathology-free. This may be especially problematic for diseases like AD where, due to a combination of high prevalence and a long prodromal period, a large fraction of individuals are likely to have presymptomatic pathology. Admittedly, this is much less of a problem with PD because of the relatively low prevalence of PD within the elderly population. Nevertheless, the difficulty of obtaining pathology-free controls would be expected to hinder biomarker discovery efforts as well as the possibility of achieving pre-symptomatic disease detection.

To investigate strategies that may aid in compensating for this inherent study limitation, we tested the effects of purposely adding a subset of younger controls to the control subject pool on diagnostic accuracy. We predicted that adding truly pathology-free controls (albeit younger and non-age-matched) to the control subject group should improve diagnostic outcome by emphasizing the non-pathology features of autoantibody profiles common to both groups. For a disease with a relatively low prevalence, such as PD, we speculate that the number of compensatory younger controls added should be relatively low—in this case perhaps no more than 5% of the total control population. However, for diseases with a much higher prevalence and longer prodromal period, such as AD, the percentage of compensatory younger controls added should be higher.

Surprisingly, in most blood-based biomarker discovery studies completed thus far for various diseases, the biomarkers showing the greatest utility generally do not include those expected based on their presumed role in the pathology [21,29,30]. In the present study, biomarker selection was dependent solely on prevalence differences between early-stage PD and controls, so as not to be

influenced by previously reported biomarker candidates. In fact, many of the 50 PD autoantibody biomarkers chosen as the top differentiators for early-stage PD follow this trend, with no obvious or expected PD-related standouts such as αlpha-synuclein. Instead, the most useful PD biomarkers selected here include some with a well-known neuronal connection, while others represent a wide variety of cellular functions and diverse molecular pathways. Among the former, microtubule-affinity regulating kinase 1 (MARK1) is a mediator of microtubule stability and is thought to be involved in neuronal migration and differentiation through phosphorylation of tau and other microtubule-associated proteins [31]. Hyperphosphorylation of tau, leading to the production of neurofibrillary tangles, has been identified as a pathological hallmark of neurodegenerative diseases such as AD and, more recently, in PD [32,33]. Interestingly, two paralogs of MARK1, brain specific kinase-1 (BRSK1), and microtubule-affinity regulating kinase 4 (MARK4) were also identified as important differential biomarkers in our diagnostic panel, suggesting a correlation to a largely unexplored pathway that could be involved in the early stages of PD pathology and progression. A 2011 study by Pickens et al. has demonstrated increased levels of the proinflammatory cytokine C-C motif chemokine 19 (CCL19) in synovial fluid from patients with either rheumatoid arthritis or psoriatic arthritis relative to controls [34]. Increased levels of another proinflammatory cytokine, interleukin 20 (IL20), have also been documented in patients with both rheumatoid arthritis and psoriasis [35,36]. Several other biomarkers identified here have been implicated in the regulation of the cell cycle, transcription and translation, as well as GTPases, RNA, and other small molecules. In addition to the biomarkers described above, a subset of the 50 identified PD biomarkers has yet to be characterized and their functions currently remain unknown.

In conclusion, we describe panels of PD autoantibody biomarkers that can differentiate early-stage PD subjects from age-matched controls, within the population studied, with a diagnostic accuracy of 87.9% using a minute volume of serum and human protein microarrays as a diagnostic platform. Because the subjects used here were diagnosed with early-stage PD with 90% confidence, it is encouraging that our overall diagnostic accuracy comes close to this 90% value. Thus, we anticipate an increase in overall accuracy when testing PD subjects with initial diagnostic confidence values approaching 100%, and such studies are currently being planned. Furthermore, we show that these panels can distinguish early-stage PD subjects from those with more advanced disease as well as differentiate them from other neurodegenerative and non-neurodegenerative diseases with high accuracy, again, within the study population. Further verification studies will be necessary to confirm comparable diagnostic accuracies in the broader population. The development of a sensitive and specific, blood-based diagnostic test for early-stage PD could have a profound clinical impact on the early treatment of PD patients who currently rely on symptoms alone for diagnosis. The use of these PD autoantibody biomarkers could fundamentally change the way PD progression is monitored in trials of potential therapies.

Author contributions

CD and RN wrote the first draft of the manuscript. Microarrays were processed by CD, NKA, GG, AS, EG, MH, and EN. All authors contributed to manuscript writing/revision. Statistical analysis was performed by CD, EN, MH and UT. DATATOP serum samples were provided by the Parkinson's Study Group.

Competing interests

The authors have the following competing interests: R. Nagele has received research funding from the Michael J. Fox Foundation, the Osteopathic Heritage Foundation, GlaxoSmithKline, the Foundation Venture Capital Group, and the Boye Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. R. Nagele is also Co-Founder of Durin Technologies, Inc., serves as its Chief Scientific Officer and has received consulting fees. He may accrue revenue in the future based on patents submitted by Rowan University wherein he is a co-inventor. B. Belinka is also Co-Founder of Durin Technologies, Inc., serves as its Chief Executive Officer and has received consulting fees. He may accrue revenue in the future based on patents submitted by Rowan University. A patent has been submitted for the PD autoantibody biomarker panel. There are no marketed products to declare.

Acknowledgments

The authors would like to thank Gerald Carey and Thuy Vo for their help with Figure 2. This study was supported by the Michael J. Fox Foundation and the Osteopathic Heritage Foundation. The DATATOP study was supported primarily by Public Health Service grant NS24778 from the National Institutes of Health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2015.09.010.

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